THE OCCURRENCE AND POSSIBLE BIOSYNTHETIC SIGNIFICANCE OF 3-HYDROXY-β-ZEACAROTENE IN A *FLAVOBACTERIUM* SPECIES

F.J. LEUENBERGER and A.J. SCHOCHER
Department of Vitamin and Nutritional Research, F. Hoffmann-La Roche & Co. Ltd., 4002 Basle, Switzerland

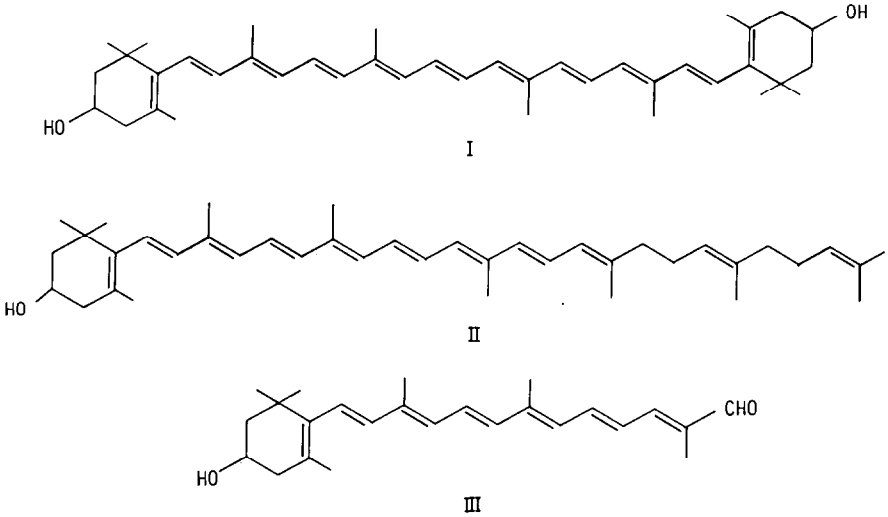
and

G. BRITTON and T.W. GOODWIN
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, England

Received 27 April 1973

1. Introduction

The Gram-negative *Flavobacterium* R-1560 [1] owes its very deep orange-brown colour to the presence of large amounts of the carotenoid zeaxanthin (β,β-carotene-3,3′-diol, I). In addition to zeaxanthin, a wide range of other carotenoids is present, in relatively small amounts [2]. Because of their unusual chromatographic properties and electronic absorption spectra, two of these compounds have been studied further, and their identification as 3-hydroxy-β-zeacarotene (7′,8′-dihydro-β,ψ-caroten-3-ol, II) and 3-hydroxy-12′-apo-β-caroten-12′-al (III) is reported. The natural occurrence of these carotenoids has not previously been described, and the possible importance of 3-hydroxy-β-zeacarotene in relation to the biosynthesis of zeaxanthin in *Flavobacterium* R-1560 is discussed.



Schemes I, II and III.

2. Methods

Flavobacterium R-1560 was grown aerobically, in the light, in a liquid medium containing glucose (1%), tryptone (1%), yeast extract (1%), $MgSO_4 \cdot 7H_2O$ (0.5%) and NaCl (3%). The pH was adjusted to 7.2 before autoclaving. Cultures were grown in 100 ml medium in 500 ml conical flasks modified by three or four indentations to act as baffles to increase aeration of the cultures, which were incubated for 48 hr at 28°C in a rotary shaking incubator.

After harvesting by centrifugation, the cells were frozen and then thawed and mixed with a little water. The slurry was poured into cold (−40°C) acetone, with vigorous stirring. The cell debris was removed by filtration and re-extracted with acetone and methanol until colourless. The combined filtrate (deep orange) was evaporated to dryness under reduced pressure on a rotary evaporator below 45°C. The residue was dissolved in light petroleum (b.p. 45°C) and the zeaxanthin that crystallized from this solution was removed by filtration. The mother liquor was evaporated and chromatographed on a column of neutral alumina (activity grade 3). After elution of less polar material with 20% diethyl ether in light petroleum, a fraction was eluted with 60% diethyl ether in light petroleum. This fraction, containing monohydroxycarotenoids, was chromatographed on thin layers of Silica gel G, with 50% diethyl ether in light petroleum as developing solvent. The two main bands, A (R_f 0.5) and B (R_f 0.3) were each rechromatographed on thin layers of MgO-Kieselgur G (1:1) with 30% acetone in light petroleum as developing solvent. In this system, A separated into an orange-yellow band (R_f 0.7), later identified [2] as β-cryptoxanthin (β,β-caroten-3-ol) and a lemon-yellow band (R_f 0.5). The latter, now identified as 3-hydroxy-β-zeacarotene (II), was further purified by TLC on Silica gel G with 40% diethyl ether in light petroleum as developing solvent, before determination of its electronic absorption, proton magnetic resonance and mass spectra. On the MgO-Kieselgur G system, B also separated into two orange-yellow bands, the lower one (R_f 0.2) later being identified [2] as β-citraurin (3-hydroxy-8′-apo-β-caroten-8′-al). The upper band (R_f 0.4), now identified as 3-hydroxy-12′-apo-β-caroten-12′-al (III) was further purified by TLC on Silica gel G with 60% diethyl ether in light petroleum before determination of its electronic absorption and mass spectra.

The acetate of II was prepared by the acetic anhydride–pyridine method [3] and purified by TLC on Silica gel G with 12% diethyl ether in light petroleum as developing solvent.

Electronic absorption spectra were determined, in ethanol, in a Unicam SP800 or Beckman DB-G spectrophotometer.

Mass spectra were determined with an A.E.I. MS9 or MS12 instrument at an ion source temperature of 220°C, and ionizing voltage 70 eV, through the courtesy of Dr. W. Vetter, Mr. W. Meister and Mr. J. Ireland.

90 MHz Fourier proton magnetic resonance spectra were determined in $CDCl_3$ on a Bruker Fourier Transform spectrometer, type HX 90/FFT with Fabritek computer 1083, through the courtesy of Dr. G. Englert.

3. Results and discussion

Two carotenoids with unusual chromatographic properties and electronic absorption spectra were isolated from *Flavobacterium* R-1560, and have been characterized as 3-hydroxy-β-zeacarotene (II) and 3-hydroxy-12′-apo-β-caroten-12′-al (III).

The former had an absorption spectrum identical to that of β-zeacarotene, with λ max (ethanol) at 405,428,453 nm, but had the chromatographic polarity of a monohydroxycarotenoid. This was confirmed by the formation of an acetate under standard conditions. The mass spectrum had the parent ion, M^+ at *m/e* 554 (intensity 36%) as expected for 3-hydroxy-β-zeacarotene ($C_{40}H_{58}O$). The presence of a strong ion at *m/e* 417 (7%, M-137, metastable at *m/e* 314; $417^2/554 = 313.9$) due to cleavage of the *bis*-allylic C-7′,8′ bond adjacent to the main polyene chromophore was consistent with the presence of the 7′,8′-dihydro acyclic end group as in β-zeacarotene [4] and also showed that the hydroxyl group was not located in this part of the molecule. The presence of the hydroxyl group was confirmed by the ion at *m/e* 536 ($M-H_2O$), and the low intensity of this (2%) indicated that the hydroxyl group occupied a non-allylic position, and was typical of a 3-hydroxy-β-ring system.

The absence of ions at *m/e* 498 (M-56) and *m/e* 469 (M-85) showed that no α-ring or hydroxylated acyclic end group was present.

The electronic absorption and mass spectra were thus consistent with the structure 3-hydroxy-β-zeacarotene (II) for this compound, and this was confirmed by the proton magnetic resonance spectrum. This had signals at 1.07 ppm (6 protons) and 1.74 ppm (3 protons) identical to those produced by the C-1 and C-5 methyl groups of zeaxanthin [5] which therefore confirms the presence of the 3-hydroxy-β-ring. Signals at 1.68 ppm (3 protons, C-1′ methyl), 1.62 ppm (6 protons, C-1′ and C-5′ methyls) and 1.86 ppm (3 protons, C-9′ methyl) were also obtained. These are virtually identical to the signals produced by the methyl groups in β-zeacarotene [4]. The "in-chain" methyl groups at C-9, 13, 13′ gave a 9-proton signal at 1.96 ppm. The PMR spectrum thus confirmed the structure as 3-hydroxy-β-zeacarotene (II).

The second compound had a rounded absorption spectrum, with λmax (ethanol) at 407 nm, which on reduction with $NaBH_4$ shifted rapidly to 355, 377, 393 nm. From this and its chromatographic properties, which indicated the presence of a hydroxyl group, this compound was identified as 3-hydroxy-12′-apo-β-caroten-12′-al (III). The mass spectrum, which had the parent ion M^+ at *m/e* 366 (100%), and fragment ions at *m/e* 351 (2%, M-CH_3) and 348 (2%, M-H_2O) was consistent with this structural assignment.

The natural occurrence of III has not previously been reported. The biochemical significance of its presence is not known, but it appears to be present in old rather than young cultures, and may therefore be a partial breakdown product of zeaxanthin.

3-Hydroxy-β-zeacarotene (II) has not previously been described but has now also been isolated from a mutant of the green alga *Scenedesmus obliquus* which accumulates ζ-carotene as its major carotenoid (R. Powls and G. Britton, unpublished results).

The presence of II is interesting from a biosynthetic point of view. It is generally accepted that hydroxylation occurs at a late stage in carotenoid biosynthesis, e.g. zeaxanthin is produced by hydroxylation of β-carotene. In *Flavobacterium* an alternative pathway, lycopene → rubixanthin → zeaxanthin is also possible [6]. The isolation of II shows that cyclization and hydroxylation can occur at an earlier stage, i.e. at the neurosporene level of desaturation, and may indicate the operation of another alternative pathway, neurosporene → II → rubixanthin → zeaxanthin. Alternatively, it may indicate that the cyclizing and hydroxylating enzymes act on the carotenoid half-molecule, when this has reached the required level of desaturation, regardless of the nature of the other end of the molecule.

Acknowledgements

T.W.G. and G.B. thank F. Hoffmann-La Roche & Co. Ltd., and the Science Research Council for financial support.

References

[1] A.J. Schocher and F.J. Leuenberger, in preparation.
[2] F.J. Leuenberger, A.J. Schocher, G. Britton and T.W. Goodwin, in preparation.
[3] G. Britton and T.W. Goodwin, in: Methods in enzymology, Vol. 18C, eds. D.B. McCormick and L.D. Wright (Academic Press, New York, 1971) p. 654.
[4] B.C.L. Weedon, Fortschr. Chem. Org. Naturst. 27 (1969) 81.
[5] W. Vetter, G. Englert, N. Rigassi and U. Schwieter, in: Carotenoids, ed. O. Isler (Birkhauser Verlag, Basel, 1971) p. 189.
[6] J.C.B. McDermott, A. Ben-Aziz, R.K. Singh, G. Britton and T.W. Goodwin, Pure Appl. Chem., in press.